CONCISE REPORT

Granuloerythropoietic Colonies in Human Bone Marrow, Peripheral Blood, and Cord Blood

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Colonies that contain granulopoietic and erythropoietic cells can be grown in specimens of human bone marrow, peripheral blood, and cord blood. Growth of these colonies is promoted by media conditioned by leukocytes in the presence of phytohemagglutinin (PHA-LCM) and the addition of erythropoietin on days 4 or 5 to the cultures. Sedimentation velocity profiles for these granuloerythrocytic colonies suggest their origin from single cells (CFU-G/E) rather than from doublets or clumps. This hypothesis is supported by cocultivation of male and female specimens. Cells in granuloerythrocytic colonies that developed in such mixing experiments were either uniformly female by Y-chromatin analysis or contained Y-chromatin bodies in both the granulocytic and erythroid cells.

INVESTIGATIONS of regulatory events in early human hemopoiesis have been greatly limited by the lack of assays for pluripotent progenitors equivalent to the spleen colony assay in the mouse.[1] However, colonies that contained more than one lineage of differentiation have been observed in cultures of of murine hemopoietic cells,[2,3] indicating that culture methods provide a reasonable approach for the development of assays for pluripotent precursors in man.

We report experimental conditions that support growth of granuloerythrocytic colonies (CFU-G/E) in specimens of human bone marrow, peripheral blood, and cord blood.

MATERIALS AND METHODS

Patients. Peripheral blood samples from eight healthy volunteers and one sample of cord blood were collected into heparinized syringes. Mononuclear cells of density less than 1.077 g/ml were separated by centrifugation in Ficoll-Hypaque.[4] Bone marrow specimens from 19 patients were obtained as part of the investigation for lymphoma, Hodgkin disease, carcinoma of the lung, acute leukemia in partial remission, and 2 patients in the stable phase after bone marrow transplantation. With the exception of those of 2 patients with acute leukemia, the bone marrow samples were not involved with disease and peripheral blood parameters were found to be within normal limits. The bone marrow samples were centrifuged at 150 g for 10 min and nucleated cells harvested from the buffy coat.

Preparation of PHA-LCM. Media conditioned by peripheral blood leukocytes of normal individuals in the presence of 1% phytohemagglutinin (PHA-LCM) were prepared.[5]

Culture conditions. Nucleated cells at concentrations of $1.5-3 \times 10^5$/ml were mixed with 5% PHA-LCM, 30% fetal calf serum (FCS), alpha medium (Flow Laboratories) without phenol red, and methylcellulose as viscous support in the final concentration of 0.9%. A volume of 0.9 ml of this mixture was placed in 35-mm Petri dishes (Lux) in 4–10 replicates and incubated at

37°C in humidified air with 5% CO_2 for 4-5 days as previously described.[6] At that time 2.5 units of erythropoietin (Connaught, step III) in 0.1 ml alpha medium without Phenol red were distributed over the whole plate by micropipetting. After subsequent incubation for a further 9-10 days, cultures were examined at 30x magnification and classified morphologically. Individual colonies were picked from the plates, placed into 0.2 ml of a 1% solution of bovine serum albumin in phosphate-buffered saline, and spun onto glass slides using a Shandon cytocentrifuge. The air-dried slides were fixed twice with cold 95% ethanol and further characterized by special staining procedures.

Staining procedures. Hemoglobin-containing cells in individual colonies were identified by benzidine stain[7] or immunofluorescence using antisera reactive against adult and fetal hemoglobin.[6] Granulopoietic cells were recognized by staining for myeloperoxidase[7] or chloroacetate esterase.[8]

Staining with atebrine and analysis by fluorescence[9] was used to identify Y-chromatin bodies in cells of individual colonies. Fluorescent Y-bodies can be identified in 60%-70% of male cells, while cells of female origin are uniformly negative.

Analysis of sedimentation velocity. Mononuclear cells were obtained from peripheral blood of normal individuals by Ficoll-Hypaque density centrifugation and analyzed by sedimentation velocity using the Staput method.[10] A constant volume of each fraction was cultured as described above, and each culture was assessed for erythroid bursts and granulocytic and granuloerythrocytic colonies.

RESULTS

Description of granuloerythrocytic colonies. When samples of human bone marrow, peripheral blood, and cord blood were cultured for 4-5 days in the presence of PHA-LCM, a number of clusters and small colonies developed. Some of these matured into granulocytic colonies after further incubation for 9-10 days. If erythropoietin was added to the cultures not later than day 4 or 5, erythroid bursts were seen. In addition, colonies were observed regularly that resembled erythroid bursts in their lobulated appearance but displayed heterogeneity with respect to the distribution of cells with red color typical of hemoglobin. Cells in some areas appeared predominantly red, while cells in other areas of the same colony lacked this typical feature of erythropoietic cells (Fig. 1 A). Hemoglobin analysis and staining for myeloperoxidase or chloroacetate esterase on individual colonies showed the coexistence of erythroid and granulocytic cells (Figs. 1 B, 1 C).

The development of these granuloerythrocytic colonies depended upon the presence of PHA-LCM, rather than conventional LCM,[11] and the addition of erythropoietin.

Frequency of granuloerythrocytic colonies. The plating efficiency was assessed for a minimum total number of 6×10^5 nucleated cells. To reduce the chance of overlapping colonies, this number was achieved by plating $1.5-3 \times 10^5$ nucleated cells in multiple dishes. For two patients CFU-G/E were linearly related to cell number over this range.

In 15 of 19 bone marrow specimens CFU-G/E were observed at a frequency of approximately 1 per $2-3 \times 10^5$ nucleated cells (Fig. 2). A similar frequency was obtained for six of eight peripheral blood samples from normal individuals. Examination of one cord blood sample yielded a frequency of 1 CFU-G/E per 2×10^5 nucleated cells.

Sedimentation velocity distribution of CFU-G/E. Mononuclear cells from peripheral blood of a normal individual were analyzed by sedimentation velocity. A single peak for CFU-G/E with a sedimentation velocity of 4-4.5 mm/hr
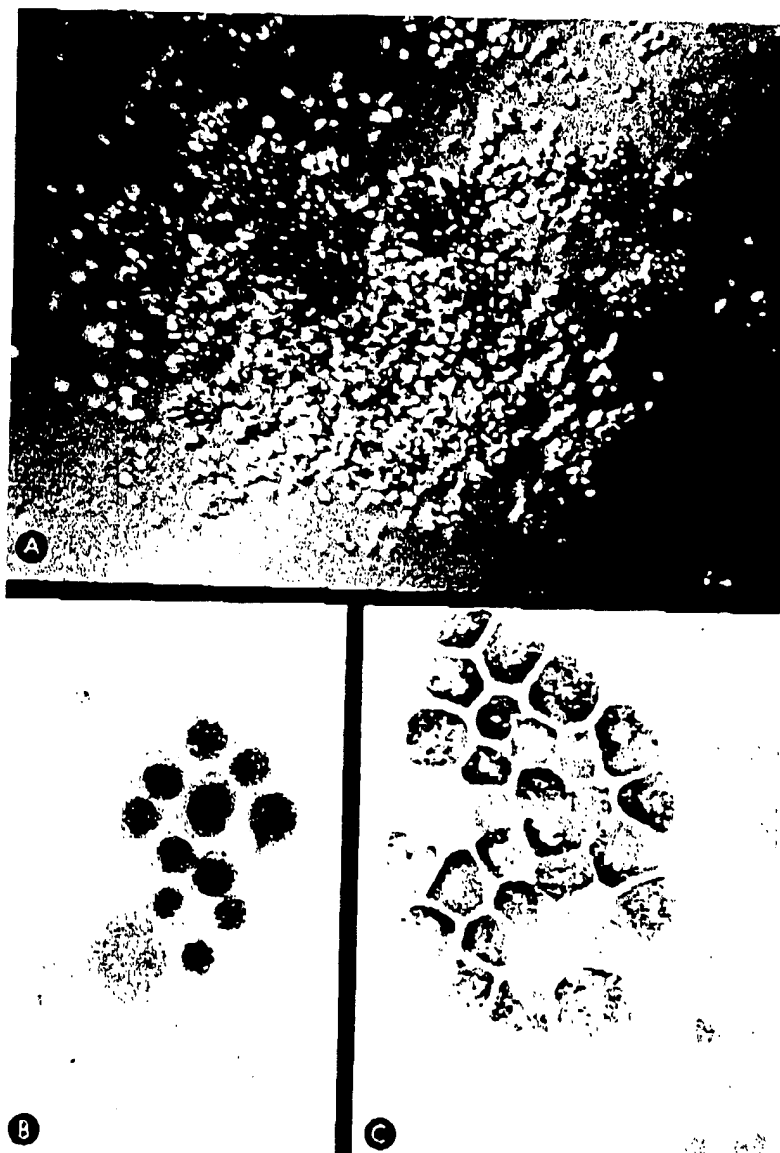
Fig. 1. (A) Granuloerythropoietic colony observed at 80× magnification. Darker areas are composed of hemoglobin-containing erythroid cells. (B) Group of erythroid cells in granuloerythrocytic colony prepared by cytocentrifugation. Wright stain. (C) Myeloperoxidase-positive granulopoietic cells in granuloerythrocytic colony prepared by cytocentrifugation.

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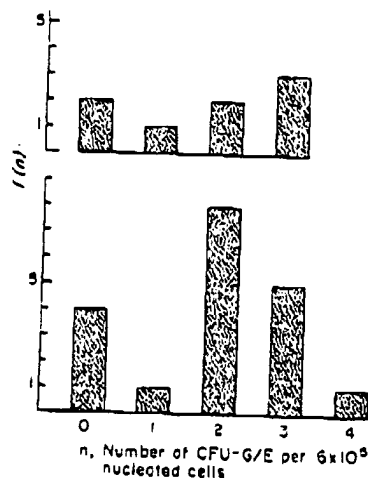Fig. 2. Frequency distribution of granuloerythrocytic colonies in peripheral blood and bone marrow.

n, Number of CFU-G/E per 6×10^5 nucleated cells

was established (Fig. 3). The distribution profile was encompassed by profiles obtained for BFU-E and CFU-C and did not have the greater sedimentation values expected for cell doublets or larger clumps.

Granuloerythrocytic colonies in cocultures of cells of male and female origin. The question of whether or not granuloerythrocytic colonies originate from BFU-E and CFU-C seeded in close proximity in the culture was approached by cocultivation of male and female cells from bone marrow or peripheral blood. Granuloerythrocytic colonies were analyzed for the presence or absence of Y-chromatin bodies. In all three experiments (Table 1) a total of 14 female colonies was observed, with the Y-chromatin absent in every cell; 11 colonies were found
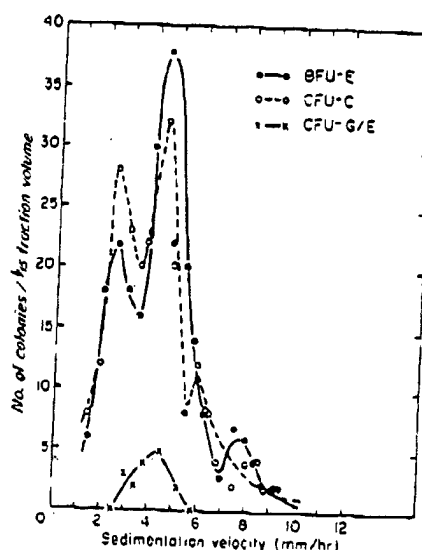

Fig. 3. Sedimentation velocity profiles for CFU-G/E, CFU-C, and BFU-E in peripheral blood.

Table 1. Y-Chromatin Body Analysis in Mixed Colonies Grown in Cocultures of Male and Female Bone Marrow (BM) and Peripheral Blood (PB) Cells

Sex	Cell Source	Total No. of Nucleated Cells ($\times 10^6$)	No. of Mixed Colonies			
			Control	Coculture*	Y-Chromatin Positive	Y-Chromatin Negative
M	BM	1.2	6			
F	BM	1.2	6	11	5	6
M	BM	1.2	4			
F	BM	1.2	4	6	2	4
M	PB	1.5	4			
F	PB	1.5	7	8	4	4

*Cocultivation of the same number of cells for each individual as plated in control plates.

that contained Y-chromatin bodies in 60%-70% of cells. This frequency is identical to the frequency of Y-chromatin bodies in cells of each granuloerythrocytic colony of male origin grown in control cultures. In mixed cultures Y-chromatin bodies were seen in both erythropoietic and granulopoietic cells.

DISCUSSION

Granuloerythrocytic colonies derived from human bone marrow, peripheral blood, and cord blood were described. Their growth depended upon the presence of PHA-LCM in the cultures, and the addition of erythropoietin on days 4 or 5 of culture was required for their final development.

Attempts were made to identify the origin of granuloerythrocytic colonies. The results obtained by sedimentation velocity analysis support the view that these colonies are derived from single cells. The profile for granuloerythrocytic colonies remains within the distribution for BFU-E and CFU-C, and they did not sediment as fast as doublets. The cocultivation of male and female hemopoietic cells yielded appropriate proportions of male and female granuloerythrocytic colonies, making it unlikely that these colonies are generated simply by overlap of colonies derived from BFU-E and CFU-C.

Both observations strongly suggest that pluripotent progenitors (CFU-G/E) are responsible for the development of granuloerythrocytic colonies.

Similar colonies have been reported for murine cells by a number of investigators. McLeod et al.[2] and Hara and Ogawa[12] described mixed erythromegakaryocytic colonies. Johnson and Metcalf[3] reported colonies that contained erythroid, granulopoietic, and megakaryocytic components when murine fetal liver cells were grown with media conditioned by murine spleen cells in the presence of pokeweed mitogen but without erythropoietin.

The availability of an assay for pluripotent progenitors will provide a tool to investigate regulatory control mechanisms responsible for commitment towards erythropoiesis or granulopoiesis. Furthermore, the availability of this assay may be instrumental in the study of the relationship of CFU-G/E and pluripotent stem cells. Properties like self-renewal and cycle state will be important for such characterization. The relevance of this assay will be established by studies of diseases such as polycythemia rubra vera or chronic myelogeneous leukaemia, both known to originate in pluripotent stem cells.[13,14]

ACKNOWLEDGMENT

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